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MOLECULAR BIOLOGY OF THE EARLY MOUSE EMBRYO

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ABSTRACT

The transition from maternal to embryonic control of development in the early mouse embryo occurs during the 2-cell stage. By the 2-cell stage, all classes of RNA are transcribed from the embryonic genome. Most of the changes in protein synthetic pattern that occur during the first cleavage are post-transcriptionally regulated. Later events, including compaction and blastocyst formation, require transcription from the embryonic genome, but some elements of post-transcriptional regulation are also involved. The result of the preimplantation developmental period is the formation of the blastocyst with two distinct cell types, the trophectoderm and inner cell mass cells. Each cell type is committed to a different developmental pathway and exhibits specific patterns of protein synthesis and DNA methylation.

INTRODUCTION

In the past decade, significant progress has been made in the manipulation of reproduction and development in mammalian species. Artificial insemination and embryo transfer are now widely used in the livestock industry and in the treatment of human infertility. While the length of time a mammalian embryo spends in the preimplantation period and the cell number achieved prior to implantation varies from one species to another, early development in every case is characterized by the formation of a blastocyst containing two morphologically and biochemically distinguishable cell types. On this basis the mouse is a convenient and inexpensive model for basic research. Moreover, genetic uniformity and the ability to use genetic approaches are important considerations in any biological study. This consideration is satisfied in mice by the many inbred strains or F1 hybrids available.

The acquisition of the background knowledge and technical achievements (see Daniel, 1971; Daniel, 1978) necessary for experimental manipulation of mammalian eggs and embryos has largely occurred within the mouse system. For example, it was in the mouse that it was determined that early blastomeres are totipotent (Tarkowski and Wroblewska, 1967) and that parthenogenetically activated eggs can develop up to post-implantation stages (Graham, 1970; Tarkowski *et al.,* 1970; Surani and Barton, 1983). Similarly, the first reliable *in vitro* culture methods (Brinster, 1963; Whittingham, 1971) and embryo freezing and thawing techniques (Whittingham *et al.,* 1972) were developed for mouse embryos. Successful constructions of chimaeric mouse embryos of tetraparental heritage have been included in the developments (Mintz, 1965). Other experimental manipulations for nuclear transfer (Surani and Barton, 1983; McGrath and Solter, 1984) and gene transfer by microinjection (Gordon and Ruddle, 1981; Costantini and Lacy, 1981; Wagner *et al.,* 1981) have also been pioneered in the mouse system. Elegant experiments with appropriate gene constructs to study expression of exogenously supplied genes ("transgenes") have followed (Palmiter *et al.,* 1982; Krumlauf *et al.,* 1985). All of these recent developments have

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depended on general knowledge about macromolecular synthesis and gene expression in the egg and early embryo.

This review begins with an outline of general procedures for handling mice, recovering early embryos, and assessment of the major morphological changes accompanying development to the blastocyst stage. An evaluation of maternal control of early development is made prior to consideration of transcription from the embryonic genome. Patterns of protein synthesis influenced by post-transcriptional processes during maternal control of the first cleavage are included along with a summary of protein synthetic activity dependent upon transcription of template RNAs from the zygote genome. The review ends with a discussion of the appearance of cell-type specific patterns of protein synthesis and DNA methylation at the blastocyst stage. Where possible, relevant comparisons to parallel developmental features in eggs and embryos of the well-studied marine organisms and other comparative systems have been included.

Obtaining Eggs and Embryos

Successful embryological studies, especially at the molecular level, require a large supply of eggs and embryos. In outbred strains of mice, natural cycles and mating lead to the generation of 12 to 14 embryos per female. Inbred strains, which are less vigorous in reproductive capacity, have smaller litter sizes. Hence, in many laboratories, female mice are hormonally stimulated to cause an increase in the number of eggs that are ovulated. Hormonal manipulation also confers the further advantage of being able to regulate the time of ovulation (within certain limits) such that the collection of eggs or embryos at specific stages of development coincides with work schedules and convenience of the investigator.

Procedures for the superovulation of mice and the timing of ovulation and mating have been described by Gates (1971) and Whitten and Champlin (1978), respectively. The highest yield of eggs (average of 89.5 per female; Gates, 1971) is obtained when prepuberal females of three weeks of age are superovulated, because this is a time when a wave of follicle maturation is occurring at the ovarian level. The immature mouse cannot, however, be used to derive embryos that have developed *in vivo* beyond the 2-cell stage (*e.g.*, morulae or blastocysts). Normal preimplantation development probably does not occur because eggs and embryos are transported too rapidly through the oviduct to the uterus (Gates, 1971). Fertilized eggs and 2-cell embryos from appropriate strains of prepuberal females will develop to blastocysts (with normal attrition rates) if subjected to *in vitro* culture. Because of high numbers at the outset, this can be a good source of *in vitro*-derived material. In all superovulation procedures, a larger proportion of preimplantation embryos (about 12%) have sister-chromatid exchange chromosomal abnormalities than do embryos (about 3.5%) derived from normal matings (Elbling and Colot, 1985).

To obtain embryos developed *in utero,* many investigators use random-bred mice that have reached reproductive age at 7 to 8 weeks. Superovulation at this stage leads to egg yields (30 to 35) that are approximately three times that of normal cycling females. The females are stimulated by intraperitoneal injection of 5 to 10 I.U. of pregnant mare serum gonadotrophin (PMSG). This preparation has a relatively stable half-life (and therefore requires only a single injection to maintain stimulation of follicles over the 2½ day period to ovulation) and is a relatively inexpensive source of FSH (follicle-stimulating hormone) activity. Induction of ovulation is stimulated 44 to 48 hours later by the intraperitoneal injection of 5 to 10 I.U. of hormone with LH (luteinizing hormone) activity. The usual commercial source is human chorionic gonadotrophin (HCG). Rupture of matured Graafian follicles and release of ova to

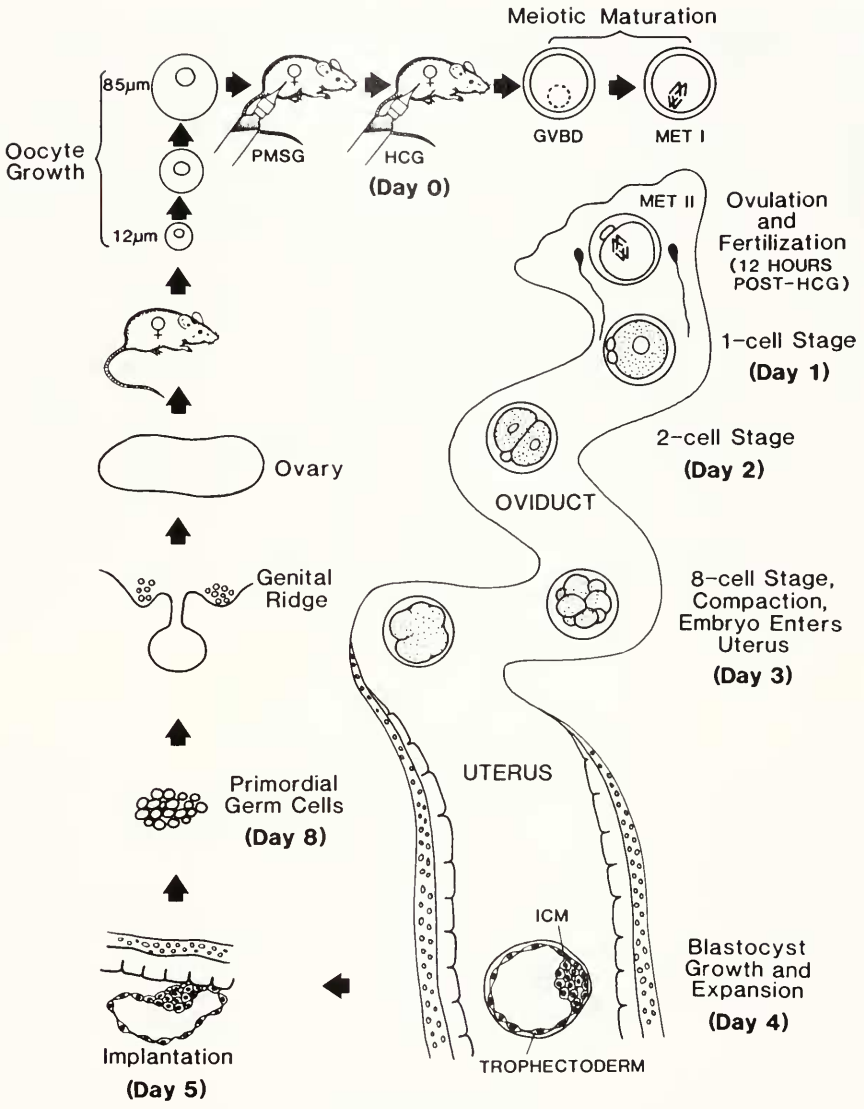

FIGURE 1. Diagrammatic outline of early development in the mouse. Abbreviations are as follows: PMSG, pregnant mare serum gonadotrophin; HCG, human chorionic gonadotrophin; GVBD, germinal vesicle breakdown; MET I, first meiotic metaphase; MET II, second meiotic metaphase; ICM, inner cell mass.

the fimbrial end of the oviduct occurs approximately 12 hours after HCG administration (Fig. 1).

The light-dark sequence is a major modulator in polyestrous mammals like mice. Endogenous LH surges lead to an ovulation time about 3 h after the mid-point of the dark period (Whitten and Champlin, 1978). Hence, it is common to employ a system in which the mouse room is maintained with 12 to 14 hours of light and 10 to 12 hours of dark with lights on at 5:00 to 7:00 hours and lights off at 19:00 hours. Injections of PMSG are often scheduled between 16:00 to 20:00 hours, and HCG is admin-

istered at 12:00 to 16:00 hours two days later. A 4:00 p.m. (16:00 hours) HCG injection leads to ovulation at about 4:00 a.m., three hours after the mid-point of a 7:00 o'clock to 7:00 o'clock dark cycle, and coincides with any endogenous LH surge. If females are placed with fertile active males at the time of the HCG injection, copulation occurs and fertilization of eggs takes place in the fimbrial end of the oviduct shortly after ovulatory release. Of course, if it is desirable to study ovulation and fertilization events during normal working hours, the cycle can be delayed by changing the dark-light cycle of the mouse room and the injection schedule.

The presence of a copulation or vaginal plug (a coagulation of seminal proteins) on the morning after mating is the criterion commonly used to infer that fertilization occurred and that preimplantation embryogenesis was initiated. With hormonal manipulation of females and selection of reproductively active males, plug rates of 75 to 80% are commonly achieved. This aids the investigator in obtaining sets of staged pregnant females from which to derive embryos at a particular stage of development. Random matings of spontaneously cycling females invariably yield low plug rates. Yet, a peak of fertile mating (up to 50% of females) often takes place on the third night after females are paired with males, due to acceleration of estrous cycling under the influence of male pheromones (Whitten and Champlin, 1978). Alternatively, females in proestrous can be identified by microscopic examination of cells from vaginal smears of mice or, for the experienced eye, the changes in vaginal size and coloration (Champlin *et al.,* 1973). These pre-selected females from a natural cycling population will yield high plug rates upon mating.

Time Course of Morphological Changes During Preimplantation Development

During early embryonic life of mice destined for production of preimplantation embryos, primordial germ cells arise and migrate via the hindgut and dorsal mesentery to the dorsal body wall and the genital ridges (Fig. 1). In female embryos oogonia transform into oocytes, which progress through early meiotic prophase during fetal life and reach the diplotene stage at about the time of birth. During prepuberal and reproductive life, sets of oocytes begin to grow from a diameter of about 12 μm to a diameter of about 85 μm over a two-week period (Fig. 1). During this period, the acellular zona pellucida that surrounds the plasma membrane of the ovulated egg (Fig. 2b) is laid down (Bleil and Wassarman, 1980). Under appropriate hormonal influence, these oocytes can be induced to undergo meiotic maturation and to be ovulated.

At the time of ovulation, the unfertilized mouse egg, like that of *Xenopus,* is arrested at second meiotic metaphase and has extruded one polar body (Fig. 2b). This state differs from that in sea urchin eggs which have completed meiosis prior to fertilization as well as the situation in *Spisula* and *Asterias,* where eggs are arrested at first meiotic prophase at the time of sperm penetration (Browder, 1984). Within 1 to 3 hours after fertilization, mouse eggs complete meiosis and generate a second polar body (Fig. 2c). The sperm head decondenses to form the male pronucleus 4 to 8 hours after sperm entry, and the female pronucleus forms between 5 to 9 hours after sperm penetration (Howlett and Bolton, 1985). The pronuclei move to the center of the egg, DNA replication occurs, pronuclei fuse, and the first cleavage division takes place about 20 hours after the time of sperm entry (Howlett and Bolton, 1985) or roughly 32 to 34 hours after the program is initiated by administration of HCG to induce ovulation (Fig. 1). Subsequent cleavages occur at roughly 12-hour intervals but synchrony among blastomeres is lost early. It is interesting to note that the first polar body often degenerates during the first cell cycle. The second polar body becomes

aligned in the plane of the first cleavage furrow (Howlett and Bolton, 1985) and is nearly always positioned between the two blastomeres at the 2-cell stage (Fig. 2d and 2e).

Following successive cleavages to the 8-cell stage (Fig. 2h), a morphological reorganization of the embryo called compaction (Fig. 2i) occurs at about 72 hours post-HCG administration. The distinct outline of individual blastomeres is lost and cells flatten tightly against one another. Gap junctions form in concert with the tight intercellular contacts and membrane and other cellular components rearrange from a nonpolarized to a radially polarized pattern (Ducibella and Anderson, 1975; Ducibella *et al.*, 1977; Lo and Gilula, 1979; Ziomek and Johnson, 1980; Reeve and Ziomek, 1981). The development of cell contacts fixes cells in position and the polarity which occurs during compaction establishes an "inside" and "outside" orientation to blastomeres. Polarity is maintained as cell division continues in the embryo and the embryo is transported from the oviduct to the uterus (Fig. 1). Cells of the embryo tend to differentiate along different lineages depending upon their relative position as "inside" or "outside" in the compacted morula. Within the 64- to 128-cell blastocyst (fourth day of development), an outer layer of trophectoderm cells surrounds the inner cell mass (ICM) cells in the blastocyst cavity (Fig. 2l). Just prior to implantation, the ICM differentiates into primitive endoderm and primitive ectoderm. The trophectoderm and primitive endoderm gives rise to extraembryonic structures and the embryonic contribution to the placenta, whereas the primitive ectoderm becomes the embryo proper (Papaioannou, 1982; Gardner, 1982).

Maternal Messenger RNA Utilization After Fertilization

During oocyte development in the mouse, all classes of RNA are synthesized and accumulated in the egg. Polyadenylated RNA [poly(A)+ RNA], as a marker of putative mRNA, also accumulates and has a high stability. The information available on RNA and protein synthesis during oogenesis in the mouse will not be reviewed here because it has been covered recently in other publications (Bachvarova, 1985; Schultz, 1986). Suffice it to say that during oocyte growth and meiotic maturation, several changes in the two dimensional patterns of newly synthesized protein occur, and during meiotic maturation about half of the accumulated polyadenylated RNA becomes either deadenylated or degraded (Bachvarova, 1985). The newly ovulated mouse egg contains about 23 ng of proteins (Brinster, 1967), between 0.35 to 0.50 ng of total RNA (Olds *et al.*, 1973; Piko and Clegg, 1982), and about 20 pg of poly(A)+ RNA (Piko and Clegg, 1982).

The first cleavage of the mouse zygote is controlled largely, if not exclusively, by informational macromolecules accumulated in the egg. From the 2-cell stage onward, transcription from the zygote genome is necessary for normal development to continue. These conclusions are derived from several lines of experimentation. First, 30 to 40% of the bulk of maternal RNA (Bachvarova and De Leon, 1980), 70% of the total poly(A)+ RNA (Levey *et al.*, 1978; Piko and Clegg, 1982), and as much as 90% of the histone (Graves *et al.*, 1985a) and actin (Giebelhaus *et al.*, 1985) messenger RNA is degraded within the first 24 hours after fertilization as development proceeds to the 2-cell stage. In this regard it is interesting to note that exogenous globin mRNA injected into newly fertilized mouse eggs is actively translated 15 to 17 hours later but is also eliminated on a functional basis by the 4-cell stage (Brinster *et al.*, 1980). Second, while some RNA synthesis has begun within pronuclei of the mouse zygote, the rate of RNA synthesis increases markedly up to the late 2-cell stage such that the synthesis of all classes of RNA is readily detectable (Young *et al.*, 1978; Piko and Clegg, 1982; Clegg and Piko, 1983a, b). Third, a large number of changes in the

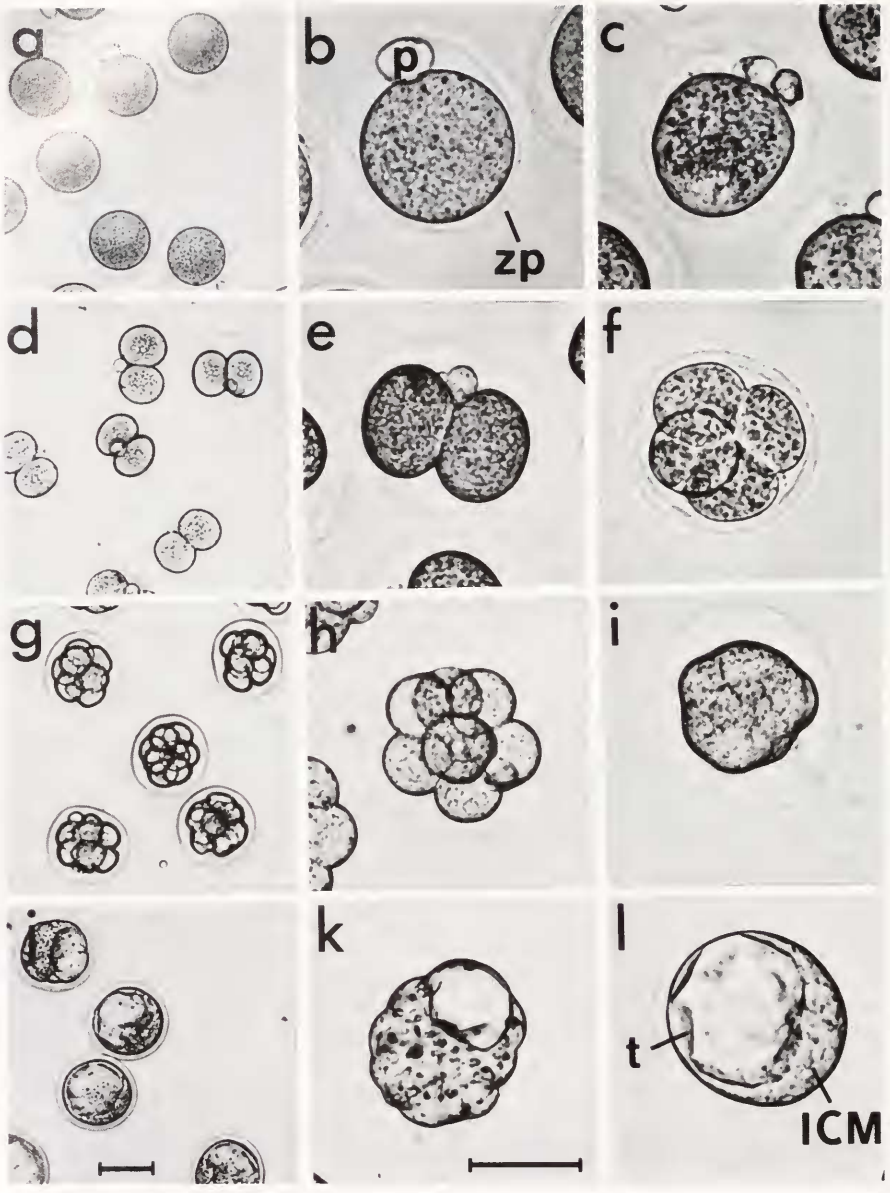

FIGURE 2. Photomicrographs of mouse eggs and preimplantation stages of development. Panels a, d, g, and j show low power views: a, unfertilized eggs at 16 hours post-HCG; d, 2-cell embryos at 42 hours post-HCG; g, uncompacted 8-cell embryos at 66 hours post-HCG; and j, blastocysts at 96 hours post-HCG. The scale bar in j is 50 μm. For higher power photographs, the scale bar in k represents 50 μm. Stages are as follows: b, newly ovulated unfertilized egg with one polar body at 14 hours post-HCG; c, fertilized egg with two polar bodies at 18 hours post-HCG; e, 2-cell embryo at 42 hours post-HCG; f, 4-cell stage at 54 hours post-HCG; h, uncompacted 8-cell embryo at 66 hours post-HCG; i, compacted 8-cell embryo at 72 hours post-HCG; k, early cavitating blastocyst at 90 hours post-HCG; l, mid-blastocyst stage at 98 hours post-HCG. Abbreviations are as follows: p, polar body; zp, zona pellucida; ICM, inner cell mass; t, trophectoderm. All photomicrographs were taken with a Zeiss IM-35 inverted phase contrast microscope.

polypeptide synthetic pattern occur as fertilized eggs develop to the 2-cell stage (van Blerkom and Brockway, 1975; Levinson *et al.*, 1978; Braude *et al.*, 1979; Howe and Solter, 1979; Cullen *et al.*, 1980). These changes appear to be regulated exclusively at the post-transcriptional level utilizing maternal components, since they also occur in physically enucleated eggs (Petzholdt *et al.*, 1980) or in eggs treated with the transcriptional inhibitor α-amanitin (Braude *et al.*, 1979; Flach *et al.*, 1982; Bolton *et al.*, 1984). Fourth, fertilized eggs treated with transcriptional inhibitors like α-amanitin and actinomycin D are markedly inhibited in development beyond the 2-cell stage (Golbus *et al.*, 1973; Warner and Versteegh, 1974). Finally, genetic variants of proteins have been used to advantage to identify paternal gene expression in embryos derived from crosses of appropriate mouse lines. Paternally derived β-glucuronidase (Wudl and Chapman, 1976) and β_2-microglobulin (Sawicki *et al.*, 1982) are both detectable by the late 2-cell stage. Since spermatozoa do not contribute mRNA to the egg, this genetic evidence, along with the biochemical studies already listed, confirms that some newly derived transcripts from the embryonic genome are utilized at least by the 2-cell stage.

Taken together, the work presented above indicates that the transition from maternal to embryonic control in the mouse embryo occurs at the 2-cell stage. The time course of this first cell division covers approximately 24 hours. In the same period of time, a fertilized *Xenopus* egg, under normal conditions, would have developed to the 10,000-cell gastrula stage and a sea urchin embryo would have developed to a 500-cell hatching blastula stage (Davidson, 1976). Similarly, the period of maternal control extends to the gastrula stage in *Xenopus* embryos and to the blastula stage in sea urchin embryos (Davidson, 1976). It is possible that the long cell-cycle for the first division in the mouse is sufficient to allow chromatin re-organization, new transcription, and early transition of genetic control from maternally derived to zygote genome-derived messenger RNA molecules.

Biochemical characterization of the maternal RNA in sea urchin and *Xenopus* eggs has revealed that as much as 70% of the mass of poly(A)+ RNA contains interspersed repetitive sequence elements (Costantini *et al.*, 1980; Anderson *et al.*, 1982). These molecules are large (often 5 to 15 kilobases in length) and similar in structure to nuclear RNA. Similar transcripts are not present in the poly(A)+ mRNA associated with polysomes of later gastrulae stages of development. This RNA with interspersed repeats is not functional when added to *in vitro* translation systems (Richter *et al.*, 1984). Analysis of poly(A)+ RNA in the mouse egg, however, suggests the absence of such non-functional transcription products containing interspersed repetitive elements (Kaplan *et al.*, 1985). One of the major repetitive elements in human and mouse DNA is the Alu sequence family (Jelinik and Schmid, 1982). RNA from full-grown oocytes and ovulated eggs does not contain any larger proportion of sequences complementary to Alu repetitive elements than does poly(A)+ RNA from differentiated cell types such as liver and brain (Kaplan *et al.*, 1985). Moreover, the molecular weight distribution of molecules containing sequences complementary to Alu DNA probes is similar in mouse egg, liver, and brain cytoplasmic RNA, a finding one would not expect if there were large unprocessed RNA transcripts in mouse egg cytoplasm (Kaplan et al., 1985).

Fertilization of the sea urchin egg triggers a series of events that results in a marked increase in the rate of protein synthesis within only a few minutes of sperm entry (Denny and Tyler, 1964; Epel, 1967). Part of this is mediated by mobilization of stored maternal mRNA components (Winkler *et al.*, 1985) into polysomes (Monroy and Tyler, 1963) and part by an increase in translation activity and elongation rate (Brandeis and Raff, 1979; Hille and Albers, 1979; Winkler *et al.*, 1985). Accompanying the increase in protein synthetic rate during the first cleavage is the doubling of

the amount of poly(A)+ RNA due to cytoplasmic polyadenylation of pre-existing mRNA species (Slater *et al.*, 1973; Wilt, 1973). The significance of this event is unknown since the rise in protein synthetic rate occurs even when cytoplasmic polyadenylation is blocked in sea urchin embryos treated with cordycepin (Mescher and Humphreys, 1974).

When [^{3}H]-adenosine is used as a precursor, a higher rate of incorporation of adenosine into poly(A) tails *versus* internal locations in the RNA of the 1-cell mouse embryo is observed (Young and Sweeney, 1979; Clegg and Piko, 1983a, b). This cytoplasmic polyadenylation process results in about a 40% increase in the number of poly(A)+ RNA molecules in the newly fertilized 1-cell zygote compared to the ovulated egg (Clegg and Piko, 1983a, b). The net increase involves polyadenylation of previously stored non-adenylated RNA molecules, but there is also a component involving degradation of some pre-existing poly(A)+ RNA. For example, actin mRNA is partially deadenylated in fertilized eggs (Bachvarova *et al.*, 1985). Thus, there appears to be a turnover in the poly(A)+ RNA population in the 1-cell embryo and this could lead to changes in the amounts of individual mRNAs and, in part, to the observed changes in protein synthetic pattern that occur in the 1-cell to 2-cell transition period. As documented earlier, 70% or more of the egg poly(A)+ RNA is eliminated through turnover by the late 2-cell stage (Piko and Clegg, 1982; Clegg and Piko, 1983a).

Post-transcriptional modification of maternal mRNA through capping has also been studied. Addition of [^{2}H]-methyl cap structures to maternally derived histone mRNAs following fertilization of the sea urchin egg has been reported (Caldwell and Emerson, 1985). The mRNAs coding for these early cleavage type histones are stored in the female pronucleus (De Leon *et al.*, 1983) and have no access to the translational machinery of the egg until released after the first nuclear division. The capping process accompanies the translation of these histone mRNAs. It has been suggested that capping of maternal mRNA may be a mechanism by which stored mRNA in sea urchin eggs is activated because it has been shown that, contrary to mammalian cells, sea urchin eggs lack the ability to initiate translation of uncapped mRNAs and have an absolute requirement for 5′ cap structures (Winkler *et al.*, 1983).

The incorporation of a low level of [^{3}H]-guanosine into 5′-terminal m^{7}G structures of RNA in 1-cell mouse embryos has also been reported (Young, 1977). Since more radioactivity was observed in m^{7}G structures than in Gp derived from internal positions, it was postulated that capping of pre-existing RNA molecules may occur in newly fertilized mouse eggs (Young, 1977). In other studies which assessed the degree of capping by translational inhibition with cap analogues and end-labeling of mRNA molecules after enzymatic removal of cap structures with tobacco acid phosphatase, no differences in unfertilized and fertilized egg mRNA were observed (Schultz *et al.*, 1980). Although post-transcriptional regulation of maternal mRNA in the 1-cell mouse embryo via capping is an appealing proposal, such a mechanism should be viewed with caution since uncapped mRNAs in mammalian cells are unstable (Banerjee, 1980) and since cap structures are required on pre-mRNAs of mammalian cells for correct splicing and excision of intervening sequences (Konarska *et al.*, 1984; Kramer *et al.*, 1984).

RNA Synthesis During Preimplantation Development

Early attempts to measure incorporation of [^{3}H]-uridine into RNA in the 1-cell mouse embryo yielded inconclusive results. Low levels of incorporation were observed, but the significance of the findings was complicated by the fact that failure to detect RNA polymerase activity in 1-cell mouse embryos (Moore, 1975) suggested

that the embryonic genome was not active until after the first cleavage. In studies on absolute rates of synthesis based on specific activities of precursor pools, Clegg and Piko (1977) discovered that [^{3}H]-adenosine was taken up and converted to ATP in mouse embryos about 1000 times more readily than the parallel conversion of uridine to UTP. Using [^{3}H]-adenosine as a labeled precursor, experiments to re-examine RNA synthesis in the 1-cell embryo were conducted (Clegg and Piko, 1983a, b). Some [^{3}H]-adenosine was observed to be incorporated into tRNA in the 1-cell embryo, but the majority was due to turnover of the 3′-terminal AMP (Clegg and Piko, 1983b). The synthesis of some heterogeneous RNA devoid of poly(A) tracts was also observed, but at a low rate (0.3 pg·cell/h). There was also a low rate of synthesis of internally labeled poly(A)+ RNA but the majority of [^{3}H]-adenosine incorporation in this class of RNA molecules was associated with turnover of 3′-terminal poly(A) tails (Clegg and Piko, 1983a, b). By the 2-cell stage, the rate of poly(A)+ RNA synthesis was measured to have increased five-fold over the 1-cell rate and ribosomal RNA synthesis was occurring at the rate of 0.4 pg/embryo/h (Clegg and Piko, 1983b).

The capacity for transcription of RNA polymerase II genes in the 1-cell fertilized egg is demonstrated conclusively by plasmid microinjection experiments. Both the herpes simplex virus (HSV) thymidine kinase (TK) gene and a hybrid gene in which the HSV TK gene is fused to a mouse metallotheionein promotor are transcribed and expressed as enzymatic activity (translation) in fertilized mouse eggs (Brinster *et al.*, 1982). In contrast to RNA polymerase type III genes (Brinster *et al.*, 1981), the TK genes are transcribed much more effectively in fertilized eggs than in growing oocytes and other pre-fertilization stages (Brinster *et al.*, 1982; Chen *et al.*, 1986). An interesting observation on RNA processing also emerges from these studies. By comparison of expression of the HSV-TK gene (no introns), the SV40 TK gene (one intron), and the chicken TK gene (six introns), the presence of introns is associated with decreased expression (Chen *et al.*, 1986). This may reflect a limited capacity for RNA splicing at this early stage of development. Nonetheless, the experiments demonstrate an enhanced capacity for transcription in fertilized eggs compared to the unfertilized ovum.

On the basis of specific activities of uridine pools, Clegg and Piko (1977) measured the absolute rates of RNA synthesis from the 2-cell stage to the blastocyst and observed a fifty-fold increase on an embryonic basis. However, if rates are calculated on a cellular basis, the value changes more modestly from about 1.25 pg/cell/h at the 2- to 4-cell stage, to 2.5 pg/cell/h in the 8-cell embryo to about 5 pg/cell/h in the blastocyst. These rates are not very much lower than the rate of total RNA synthesis (5.7 pg/cell/h) reported for exponentially growing HeLa cells (Brandhorst and McConkey, 1974). The rates are greater than those measured for heterogeneous RNA synthesis in cleavage and blastula stages of sea urchin embryos (Brandhorst and Humphreys, 1971; Wu and Wilt, 1974). In summary, all classes of RNA, including poly(A)+ RNA, are actively synthesized from the 2-cell stage and onwards throughout the preimplantation period (Levey *et al.*, 1978; Piko and Clegg, 1982).

Whereas maternal oocyte mRNA is largely degraded by the late 2-cell stage (see previous section), turnover rates of newly synthesized RNA have also been studied. The average half-life of mRNA measured in mouse morulae is 8 to 11 hours, while that in blastocysts is 14 to 26 hours (Kidder and Pedersen, 1982). As in HeLa cells (Singer and Penman, 1973) and rabbit blastocysts (Schultz, 1974), the mRNA decay profile in mouse blastocytes also has been observed to be biphasic with short-lived (about 6 hours) and long-lived (24 hours or more) components (Kidder and Pedersen, 1982). Such decay curves, however, probably represent an average of a continuum of decay of different classes of mRNA with different half-lives. The mixture of short-lived and long-lived components is consistent with studies on the continued synthesis

and disappearance of certain polypeptides when morulae are cultured to blastocyst stages in the presence of the transcriptional inhibitor, α-amanitin (Braude, 1979a, b).

One group of mRNAs that has been studied in detail in the early mouse embryo is the set of transcripts derived from histone genes. The histone genes of mammals are part of a small multi-gene family with 10 to 20 different genes for each histone protein. Four H3 genes, three H2b genes and two H2a genes have been isolated from three separate mouse genomic clones (Sittman *et al.,* 1983; Graves *et al.,* 1985b). Two of the gene clusters are localized on chromosome 13, and the third is on chromosome 3. An S1 nuclease mapping technique has been developed to measure expression of the individual genes (Graves *et al.,* 1985b). When this technique is applied to maternal mRNA derived from unfertilized mouse eggs and zygote-genome mRNA derived from the blastocyst stage, a number of changes are observed. A large amount (40 to 50%) of the histone H3 mRNA in the egg is complementary to the H3 gene located on chromosome 3 (H3.614) whereas only 14% of the histone H3 mRNA in the blastocyst is derived from the H3.614 gene (Graves *et al.,* 1985a). Similarly, nearly all the H2a mRNA in the egg is derived from the H2a.614 gene on chromosome 3, whereas only 30% of the H2a mRNA in blastocysts is complementary to the H2a.614 sequence. These and other data demonstrate that the same set of histone genes seem to be expressed in eggs and early embryos, but there are large differences in the relative abundance of certain histone mRNA types.

Changes in histone gene sets expressed during sea urchin development are also well-documented (Newrock *et al.,* 1978; Maxson *et al.,* 1983), but just as in the mouse embryo, the significance of these changes with respect to control of gene expression is not known.

Patterns of Protein Synthesis in the Early Embryo

The ovulated mouse egg contains the machinery to synthesize proteins due to accumulation of rRNA, mRNA, tRNA, and ribosomes during oogenesis (Bachvarova, 1985). However, many of the ribosomes in the egg may not be functional when judged by their ability to form initiation complexes *in vitro* with a synthetic messenger RNA (Bachvarova and De Leon, 1977). Indeed, spare translational capacity of the fertilized mouse egg for injected globin mRNA is extremely limited (Ebert and Brinster, 1983). During the first 24 hours of post-fertilization development, there is little change in net protein synthetic rate, protein turnover, or total protein content (Brinster *et al.,* 1976; Merz *et al.,* 1981). During this same interval, there is a loss of 70 to 90% of the mRNA (Piko and Clegg, 1982; Giebelhaus *et al.,* 1983). It follows that much of the mRNA in the unfertilized egg is in a form where it is not used for translation or is used at a very low efficiency.

From a qualitative point of view, the changes in the pattern of protein synthesis during the early cleavage period are very marked. In a previous section we have already documented that many of the changes in the 1-cell to 2-cell transition period are post-transcriptionally controlled. It is important to note that some of the changes in protein pattern and cytoplasmic structure (*e.g.,* mitochondrial translocation; van Blerkom and Runner, 1984) that occur up to the stage of pronuclear fusion appear to be controlled by post-translational processes such as glycosylation and phosphorylation rather than translation of stored maternal mRNA (van Blerkom, 1981, 1985; Howlett and Bolton, 1985). Other changes up to the 2-cell stage do seem to depend on sequential activation of selected mRNAs by some translational control mechanism (Braude *et al.,* 1979; Cascio and Wassarman, 1982). In summary, there are fertilization-independent, fertilization-accelerated, and fertilization-dependent changes in polypeptide synthesis during the first cleavage. Some are due to post-translational

modification, some are due to differential mRNA activation and others are due to differential polypeptide turnover (Howlett and Bolton, 1985). All of the latter mechanisms are involved in producing the well-described series of changes in polypeptide synthetic pattern that occur within a protein complex with a molecular mass of about 35,000 during the first 24 hours after fertilization (Levinson *et al.,* 1978; Braude *et al.,* 1979; Cullen *et al.,* 1980; van Blerkom, 1981; Flach *et al.,* 1982; Howlett and Bolton, 1985).

Changes in patterns of protein synthesis following fertilization or egg activation also occur in other systems. In the sea urchin embryo, there is a large increase in protein synthetic rate at fertilization (Grainger *et al.,* 1979) with accompanying changes in the qualitative pattern of protein synthesis (Evans *et al.,* 1983). For example, maternal mRNAs which code for four proteins (whose synthesis is barely detectable in the unfertilized egg) become actively translated to yield abundant products of synthesis after activation. Some of these proteins (termed cyclins) are destroyed every time the cell divides (Evans *et al.,* 1983). In addition, mRNAs for early histone variants are stored in the female pronucleus (Venezky *et al.,* 1981) but are not translated until after the first cleavage (Wells *et al.,* 1981). With continued development, a switch to late embryonic histone variants occurs (Maxson *et al.,* 1983). In the surf clam, *Spisula solidissima,* there is only a small increase in protein synthetic rate upon fertilization but a major change in the classes of proteins that are synthesized due to selective activation of maternal mRNAs also occurs (Rosenthal *et al.,* 1980; Tansey and Ruderman, 1983). Perhaps these changes are so dramatic because the *Spisula* egg has not yet undergone meiotic maturation. In any regard, striking examples of translational control of changing patterns of protein synthesis are documented in these studies.

An interesting set of polypeptides which appears at the early 2-cell stage of mouse development is a complex with approximate molecular weight of 67,000 to 70,000. Synthesis of these polypeptides is dependent upon new transcription since they do not appear in fertilized eggs cultured to the 2-cell stage in the presence of α-amanitin (Flach *et al.,* 1982; Bensaude *et al.,* 1983; Bolton *et al.,* 1984). One-dimensional peptide maps of the 68,000 (68K) and 70,000 (70K) components are not distinguishable from two heat shock proteins, hsp 68 and hsp 70 derived from cultured mouse F9 cells (Bensaude *et al.,* 1983). The developmental regulation of such heat shock or stress proteins is not unique to 2-cell mouse embryos. For example, hsp 70 mRNA accumulates in *Xenopus* oocytes (Bienz and Gurdon, 1982) but is translated in the oocyte only after heat shock treatment. After fertilization, translation of the hsp 70 mRNA cannot be induced by hyperthermia and cleavage stage embryos lack measurable hsp 70 mRNA and have no detectable hsp 70 synthesis (Bienz, 1984; Heikkila *et al.,* 1985). A number of hsp mRNAs also accumulate in *Drosophila* oocytes (Zimmerman *et al.,* 1983) and larval and pupal stages (Cheney and Shearn, 1983). It is interesting to note, however, that in spite of the developmental regulation of the hsp 68-70 genes, the cleaving mouse embryo is refractory to induction of additional hsp synthesis by exogenous stress-inducing stimuli. A heat-shock "incompetent" period, in which hsp 68-70 synthesis is not induced in response to environmental stress, has also been observed prior to the blastoderm stage of *Drosophila* embryos (Dura, 1981), the blastula stage in sea urchin embryos (Roccheri *et al.,* 1981), and the mid-blastula stage in *Xenopus* embryos (Bienz, 1984; Heikkila *et al.,* 1985). The ability to respond to stress by the synthesis of hsp 68-70 is acquired by the blastocyst stage in both the mouse (Wittig *et al.,* 1983; Morange *et al.,* 1984) and the rabbit embryo (Heikkila and Schultz, 1984). Failure of induction during cleavage may be related to the presence of constitutive levels of hsp 70 protein in the early embryos (Morange *et al.,* 1984) al-

though the differential response to heat shock in the preimplantation embryo remains unexplained and awaits further study.

Although the patterns of protein synthesis during the first cell division have been shown to be derived from the translation of both maternal and zygote-genome derived templates, there is no evidence for the translation of oogenetic mRNA in the mouse embryo after the 4-cell stage. Processes such as compaction, cavitation, and blastocyst formation require transcription from the embryonic genome, although an element of post-transcriptional regulation is also involved (Kidder and McLachlin, 1985). For example, the transcription of templates necessary for the critical events in compaction of the 72-hour post-HCG 8-cell embryo are completed by the 4-cell stage (Kidder and McLachlin, 1985). Conversely, in the process of cavitation and development of the morula to the blastocyst, transcriptional and translational processes are tightly coupled (Braude, 1979a, b; Kidder and McLachlin, 1985).

From a qualitative point of view, two-dimensional gel patterns of proteins synthesized by 72-hour post-HCG morulae have been compared to those from embryos cultured *in vitro* to the blastocyst stage and to patterns from embryos arrested by culture in the presence of α-amanitin (Braude, 1979a, b). The major feature of these patterns is that the majority of the polypeptides show little change between the morula and blastocyst stages and are translated from messenger RNAs of relatively high stability, since they continue to be synthesized 24 hours later despite the presence of a transcriptional inhibitor. A small set of polypeptides (Braude, 1979b) fails to appear or to increase in intensity in the presence of the inhibitor and a similar number persists in the presence of the inhibitor when normally synthesis would have ceased prior to the blastocyst stage. The normal events ultimately do lead to the appearance of a number of specific polypeptides in both the trophectoderm and ICM cell lineages at the blastocyst stage (van Blerkom *et al.,* 1976; Handyside and Johnson, 1978; Brulet *et al.,* 1980; Howe *et al.,* 1980).

Quantitatively, the rate of protein synthesis remains at a relatively low level from fertilization to the 8-cell stage. Once the 8-cell stage is reached, there is a progressive increase in synthetic rate accompanying the transition of the morula to the blastocyst (Epstein and Smith, 1973; van Blerkom and Brockway, 1975; Brinster *et al.,* 1976; Abreu and Brinster, 1978). Ribosome numbers increase progressively with developmental stage due to active synthesis of rRNA and ribosomal proteins (La Marca and Wassarman, 1979). These rates of synthesis are sufficient for the production of about 2.5×10^6 ribosomes/embryo/h and can account for both the increase in ribosomal content and increased protein synthetic rate in the blastocyst (Piko and Clegg, 1982). An additional component may involve a shift in the pool of poly(A)+ RNA in the subribosomal fraction of morulae to the polysome fraction in blastocysts (Kidder and Conlon, 1985).

DNA Methylation in Early Development

Considerable interest has surrounded the field of DNA methylation in cell differentiation, because methylation of DNA sequences, for at least some genes in eukaryotes, is associated with inhibition of transcription (see Ehrlich and Wang, 1981, and Doerfler, 1983 for reviews). Five (5)-methyl cytosine appears as a minor base in the DNA of many organisms. It is produced by enzymatic (methylase) addition to some of the cytosine residues that are adjacent to guanine (m^5CpG) in genomic DNA. During DNA replication, the cytosine of the newly synthesized strand is usually symmetrically methylated as soon as a sequence is made. If a CpG pattern is not methylated, it will remain that way (Ehrlich and Wang, 1981). An alteration of the methylation pattern of a cell (hyper- or under-methylation) relative to a parental cell line can

potentially lead to a heritable change that will be passed on from one cell generation to the next, generating a distinct cell lineage at the DNA level.

In mammalian cells, initial studies indicated that genes were undermethylated in tissues in which they were expressed during development or cell differentiation and hypermethylated when inactive. This appears to be true for about one-third of the thirty genes analyzed to date (Kolata, 1985). In addition, many of the so-called "housekeeping genes," which are active in all cell types are undermethylated at their 5′-terminal initiation sequences. In another 20% of the genes, there is no correlation between methylation and gene activity (Kolata, 1985). Since there is a lesser degree of methylation of cytosine residues in lower vertebrates and essentially no methylation in *Drosophila* DNA (Ehrlich and Wang, 1981), association of methylation with gene expression may be restricted to mammalian cells. In addition, methylation may be associated only in a secondary way with respect to expression of genes rather than being the primary event in turning off genes. Nonetheless, interesting patterns of DNA methylation are associated with formation of the first distinct cell lineages in the mouse embryo.

At about 4.5 days of development, just before implantation of the blastocyst, ICM cells become either primitive ectoderm or primitive endoderm. Along with the trophectoderm layer, the primitive endoderm gives rise to extraembryonic structures while the primitive ectoderm contributes to the three germ layers of the embryo (Papaioannou, 1982; Gardner, 1982). On the basis of methyl-sensitive restriction endonuclease digestion patterns of mouse satellite DNA and dispersed repetitive sequence, it has been shown that these sequences are undermethylated in all derivatives of the extraembryonic lineages compared to those in primitive ectoderm or DNA of adult tissues (Chapman *et al.*, 1984). Similarly, the DNA of the trophoblast component of rabbit blastocysts has been shown to be undermethylated (Manes and Menzel, 1981). Recently it also has been demonstrated that the undermethylation of DNA in extraembryonic structures of the mouse embryo is not restricted to repetitive sequences and includes alpha-fetoprotein, albumin, and major urinary protein structural gene sequences (Rossant *et al.*, 1986). The same sequences are heavily methylated in embryonic tissues as early as 7.5 days of development (Rossant *et al.*, 1986). These findings along with other studies (Razin *et al.*, 1984; Young and Tilghman, 1984) confirm that major differences in DNA methylation occur as cell lineages are established in the early embryo.

In the process of establishing different methylation patterns in the early cell lineages, both *de novo* methylation and demethylation probably occur. In general, sperm DNA is highly methylated with respect to structural genes, although satellite DNA sequences are less methylated in sperm than in adult tissues (Waalwijk and Flavell, 1978; Sanford *et al.*, 1985). Oocyte DNA is undermethylated with respect to repetitive DNA sequences (Sanford *et al.*, 1985). Retroviral sequences introduced into the preimplantation embryo become *de novo* methylated, but sequences introduced into post-implantation stages (8-day mouse embryos) escape methylation (Jahner *et al.*, 1982). Taken together with the fact that the DNA of cleavage-stage rabbit (Manes and Menzel, 1981) and mouse embryos (Singer *et al.*, 1979) has levels of methylation similar to that of adult tissues, it is possible that the *de novo* methylation of sperm and egg DNA may occur early. If this is so, it follows that the differentiating trophectoderm and primitive endoderm lineages must undergo extensive demethylation processes. There are insufficient data to make firm conclusions about the timing of *de novo* methylation events in the embryo, and the possibility that *de novo* methylation does not occur until the primitive ectoderm lineage is established must also be entertained (Sanford *et al.*, 1985). Although it is clear that the methylation pattern of the alternate lineages is distinct, the significance of this observation with respect to

subsequent differentiation and gene expression in each line remains to be established through further research.

Concluding Remarks

Analysis of the molecular biology of the early mouse embryo is difficult because of its small size and limited numbers of available embryos. Nonetheless, the development of recombinant DNA techniques of high sensitivity recently has allowed approaches to problems of gene expression in early mouse embryos that previously could be studied only in systems where embryological material was more abundant. Using S1 mapping techniques, it has been possible to measure histone mRNA transcripts in mouse blastocysts that are present in as few as 600 copies per cell (Graves *et al.,* 1985a). *In situ* hybridization methods have been developed for sea urchin embryos that can detect mRNAs in defined cell types in as few as 50 copies or less per cell (Cox *et al.,* 1984; Angerer and Davidson, 1984). This molecular cytological approach can be applied to a small number of embryos and is potentially well-suited to investigation of early mammalian embryos. Although laborious to construct, cDNA libraries from mRNA from mouse oocytes and blastocyts have now been made (McConnell and Watson, 1986). In time these libraries will aid the identification of genes expressed at particular stages of early development.

A key feature of development in the mouse that requires future emphasis is the identification of marker genes that are activated in time and space during the period of early implantation, germ line formation in the embryo proper, and early morphogenesis and organogenesis. The techniques now exist to study patterns of gene expression during this critical phase. I purposely have not included extensive discussions of the use of genetically engineered transgenic mice in this review because, to date, this approach has yielded information primarily on tissue specificity of gene expression in fetal or adult organs. Nonetheless, expression from fusion plasmids microinjected into mouse pronuclear zygotes has also been observed in cultured preimplantation embryos (Brinster *et al.,* 1982; Pedersen and Meneses, 1985; Chen *et al.,* 1986). These approaches are applicable to gene expression studies during early development and during cell lineage formation and offer exciting prospects for elucidation of mechanisms underlying cellular commitment events in the embryo.

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